## REMARKS

Claims 1-2 and 4-20 are pending. Claims 1 and 2 are amended with the subject matter of claim 3. No new matter is added.

It will be empirically shown below (in rebuttal of the second rejection) that the use of only polar-organic-solvent or water, as disclosed in the prior art, ***cannot get the extract*** contained gnetin C which shows ***both antimicrobial and antioxidative*** activities. Rather the ***claimed mixture of both solvents*** (claims 1 and 2) and the control of temperature and time for the aging is necessary to improve solubility of the constituents and to promote reactivity of glucosidase (namely, age) to obtain the extract containing rich gnetin C.

Thus the invention as now claimed is not *prima facie* obvious in light of the cited art for the reasons below.

**Claims 2-4, and 10-15 are rejected under 35 U.S.C. 103(a) as being unpatentable over Boralle et al (Oligostibenoids from Gnetum venosum, Phytochemistry, 34 (5): 1403-1407, 1993), in view of Berry (Cyclopropene fatty acids in Gnetum gnemon (L.) seeds and leaves, Journal of the Science of Food and Agriculture, (1980) Vol. 31, No. 7, pp. 657-662).** (Office Action, page 2)

The applicants found that ***water/polar-organic-solvent ratios of the extraction solvent influenced antimicrobial and antioxidative actions of the extract*** in which ***content of the constituents having those actions was proportional to the spot area of Rf value 0.5*** (based on gnetin C). It was observed that enlargement of this spot area by the aging reduced the spot area of Rf value 0.15 (based on gnemonoside A) and the glucosidase in seeds (kernels, endosperms) concerned with conversion of the constituents (gnemonoside A to gnetin C *via* gnemonoside D). Gnemonoside A (diglucoside, freely soluble in water), gnemonoside D (monoglucoside, soluble in water) and gnetin C (aglycon, slightly soluble in water) as the major constituents were isolated by the separation and purification of the extract and examined for ***antimicrobial and antioxidative actions***.

The antimicrobial and antioxidative activities decreased in the order of gnetin C, gnemonoside D, gnemonoside A. The HPLC analysis showed that the extraction of products peeled and ground Gnetum seeds (kernel powder) with ethanol and water, respectively, resulted in low yields of these constitutions and especially very poor yield of gnetin C. In the water/polar-organic-solvent ratios of about 1:1 the extract had high content of gnetin C by acceleration of glucosidase. This phenomenon, which is new information, suggests the participation of the activation of glucosidase and the water-solubility of these constituents.

Nowhere in the cited art has it been shown that water/polar-organic-solvent ratios of the extraction solvent influenced antimicrobial and antioxidative actions of the extract in which content of the constituents having those actions was proportional to the spot area of Rf value 0.5 (based on gnetin C). Because this was not known, it cannot be concluded that the applicants merely optimizing, because the alleged optimization goal was unknown. Therefore claim 2 and claims dependent thereon are not in fact obvious in light of the cited art. This point will be empirically shown below.

**Claims 1, 5, 7-9 and 16-20 are newly rejected under 35 U.S.C. 103(a) as being unpatentable over Boralle et al (Oligostibenoids from Gnetum venosum, Phytochemistry, 34 (5): 1403-1407, 1993), in view of Berry (Cyclopropene fatty acids in Gnetum gnemon (L.) seeds and leaves, Journal of the Science of Food and Agriculture, (1980) Vol. 31, No. 7, pp. 657-662) and further in view of Iliya et al (Iliya et al, Stilbene derivatives from two species of Gnetaceae, Chem. Pharm. Bull. 50(6) 796-801 (2002)).** (Office Action, pages 5-6)

Claims 1 and 2 have been amended with the subject matter of claim 3 thus making this rejection moot with respect to claim 1 and all claims dependent thereon.

In addition to this, Iliya et al. does not search constituents in fruits of Gnetaceae which are utilized for food, but selects and extracts root of *Gnetum gnemon* and stem lianas of *G. gnemonoides* which are inedible. ***Iliya does not test*** the constituents isolated from these extracts for ***antimicrobial and antioxidative actions***, as shown in the quotation below. (Iliya Chem. Pharm. Bull. **2002**, 50, p.800 col.1 and 2). (emphasis added by underline)

**Experimental** ...

**Extraction and Isolation** The dried root of *Gnetum gnemon* (2.0 kg) and the stem lianas of *G. gnemonoides* (1.0 kg), collected in April 2001 at Bogor Botanical Garden, Indonesia, were powdered and separately extracted, successively, with aceton and methanol. The acetone extract of *Gnetum gnemon* (60 g, 3% yield) was chromatographed on silica gel and eluted with a mixture of $CHCl_3$-MeOH of increasing polarity to give 11 fractions. Compound **6** (gnetin E; 1.5 g, 0.075% yield) was obtained from fraction 3 and was purified by VLC on ODS and eluted with MeOH-$H_2O$ (1:1). Fraction 4 was subjected to VLC on ODS and eluted with MeOH-$H_2O$ (1:1) to give 10 fractions (fr. A-J). Further purification of fraction F by PTLC [Benzene : EtOAc : acetone : $H_2O$ (40 : 30 : 30 : 1)] gave **1** (gnemonol A; 20 mg; 0.001% yield). Compound **2** (gnemonol B; 97 mg; 0.005% yield) was obtained from fraction J in pure form. The acetone extract of *G. gnemonoides* (27 g; 2.7%) was also subjected to chromatography on silica gel and eluted with a mixture of $CHCl_3$-MeOH of increasing polarity to give 33 fractions. Fractions 12, 13, 14 and 15were separately chromatographed on sephadex LH-20 and eluted several times with methanol to yield compound **6** (gnetin E; 34.0 mg; 0.0034% yield), **7** (2b-hydroxyampelopsin F; 30 mg; 0.003% yield), **5** (gnetal; 3.0 mg; 0.0003% yield) and **3** (gnemonol C; 24.0 mg; 0.0024% yield), respectively. Compound **4** (gnemonoside E, 23.0 mg; 0.0023% yield) was obtained from fraction 23 by repeated chromatography of the fraction on an ODS open column eluted with a mixture of MeOH-$H_2O$ (4:6).

The constituents obtained from these materials differ from gnemonoside A, gnemonoside D and gnetin C contained in endosperms. The whole yields (underlined parts above) of the constituents from the root and the stem lianas were 0.08% and 0.0114%, respectively. ***These results correspond to the yields of the extraction of endosperms with 99% ethanol*** (run 10 in Table 1; run 1 in Table 3 – Comparison II-1).

Table 1. The influence of the concentration of ethanol and extraction temperature on aging at 50°C

| run | concn. | time | yield | yield | | | | embodiment |
|---|---|---|---|---|---|---|---|---|
| | | h | extract | Gd. A[a] | Gd. D[b] | Gn. C[c] | sum[d] | |
| 1 | 0% | 4.5 | 10.5% | 0.61% | 0.00% | 0.01% | 0.62% | |
| 2 | 10% | 4.5 | 10.2% | 0.63% | 0.00% | 0.02% | 0.65% | |
| 3 | 20% | 4.5[e] | 10.4% | 0.59% | 0.25% | 0.14% | 0.98% | I-3, II-8 |
| 4 | 30% | 4.5 | 10.6% | 0.81% | 0.71% | 0.59% | 2.11% | |
| 5 | 40% | 5.0 | 10.6% | 0.59% | 0.79% | 1.48% | 2.86% | II-2 |
| 6 | 50% | 4.5 | 12.7% | 0.73% | 0.65% | 1.29% | 2.67% | |
| 7 | 60% | 4.5 | 12.3% | 0.96% | 0.79% | 1.16% | 2.91% | |
| 8 | 70% | 4.5 | 11.6% | 1.14% | 0.85% | 0.91% | 2.90% | |
| 9 | 80% | 4.5 | 9.5% | 1.28% | 0.59% | 0.62% | 2.49% | |
| 10 | 99% | 4.5 | 4.2% | 0.02% | 0.02% | 0.03% | 0.07% | |

a: gnemonoside A b: gnemonoside D c: gnetin C d: sumention G d: sum of Gd. A, Gd. D and Gn. C
e: warmed after immersing at 15 °C over night

Table 3. The influence of the concentration of ethanol and extraction temperature on aging in 99% ethanol

| run | temp. | time | yield | yield | | | | comparison |
|---|---|---|---|---|---|---|---|---|
| | °C | h | extract | Gd. A[a] | Gd. D[b] | Gn. C[c] | sum[d] | |
| 1 | 27 | 48 | 4.8% | 0.03% | 0.01% | 0.01% | 0.05% | II-1 |
| 2 | 80 | 5 | 6.9% | 0.11% | 0.03% | 0.03% | 0.17% | II-2 |

The ***difference between the claimed invention and Iliya's paper on the isolation of the constituents in extracts can be empirically shown.*** The procedures used to show this are recited as:

**Procedures:**

The Applicant soaked the endosperms in 50% ethanol at room temperature (25°C) for 2 days and obtained the gnetum extract in 9.33% yield. The HPLC (column: ODS, mobile phase: methanol/water/acetic acid = 64/35/1, flow rate: 0.8 ml/min, detector: 320 nm) analysis of this extract showed that the extraction yields of gnemonoside A (retention time: $t_R$, 3.9 min), gnemonoside D ($t_R$, 4.5 min) and gnetin C ($t_R$, 7.4 min) were 0.47%, 0.70% and 1.48%, respectively. The isolation of these constituents was carried out in the following manner to obtain

gnemonoside A, gnemonoside D and gnetin C in 0.40%, 0.63% and 0.63% yields, respectively.

**Run 3 in table 2** The products peeled and ground gnetum seed (endosperms, 40.0 g) were soaked in 50% EtOH (120 mL) at room temperature for 2 days, and the mixture was filtered. The filtrate was evaporated *in vacuo* to obtain the gnetum extract (3.73 g, 9.33% yield). This extract was chromatographed on reversed-phase ODS eluted with 25% MeOH, 40% MeOH, and 60% MeOH to produce four fractions. The second fraction (0.63 g, 1.58% yield) was purified by Sephadex LH-20 column chromatography with 50% MeOH to give gnemonoside A (0.16 g, 0.40% yield) as a pale brown amorphous powder, $[\alpha]_{546}$ +68.3 (c 0.7, MeOH). The third fraction (0.46 g, 1.15% yield) was purified by silica gel column chromatography with $CHCl_3$/MeOH 4:1 (v/v) to give gnemonoside D (0.25 g, 0.63% yield) as fine needles. These crystals were recrystallized with 25% MeOH to yield colorless needles, mp 205–206 °C, $[\alpha]_{546}$ –65.6 (c 0.6, MeOH). The fourth fraction (0.30 g, 0.75% yield) was purified by silica gel column chromatography with $CHCl_3$/MeOH 9:1 (v/v) to give gnetin C (0.25 g, 0.63% yield) as a pale yellow amorphous powder, $[\alpha]_{546}$ –23.2 (c 0.5, MeOH).

The whole isolated yield of these constituents is 1.66%. ***This yield is 20 times (root of G. gnemon) and 146 times (stem lianas of G. gnemonoides) that disclosed in Iliya.*** The extraction and aging of the endosperms with the ***mixture of ethanol and water resulted in the unexpected improvement on the isolation yield.***

Embodiment II-4 of the instant invention is an example of acetone with which Iliya et al. extracted. The extraction and aging with 80% acetone at room temperature (30°C) over night, 60°C for 5 hours and then room temperature for 3 days gave ***the extract in 9.3% yield***. The HPLC analysis of this extract showed that the yields of gnemonoside A, gnemonoside D and gnetin C were 0.18%, 0.62% and 1.37%, respectively. These yields which differ obviously from the Iliya's art resemble the ***results of 50% ethanol extraction and aging.*** These differences are aging of endosperms in polar organic solvent-water mixtures.

**The influence of ethanol concentration, temperature and time on the aging:**

The HPLC analysis result of the extracts obtained by extracting and aging the endosperms in various ethanol-water mixtures (percent: ethanol concentration) at 45~50°C is represented in Table 1 (above). ***The increase of ethanol concentration enlarged the sum of yields of these constituents. However, the yields fell remarkably in the case of 99% ethanol (run 10).*** The yields of gnemonoside A were not appreciably altered by change of the ethanol concentration below 80%, while those of gnemonoside D and gnetin C were altered by change of ethanol concentration in the range of 20% to 80%. In particular, ***gnetin C*** was significantly ***high yield in the range of 40% to 50%.*** Although freely soluble gnemonoside A which is not eluted from endosperms with 99% ethanol cannot be hydrolyzed by glucosidase, gnemonoside A dissolving in 40~50% ethanol is hydrolyzed by stimulated glucosidase. In the case of ethanol concentration below 20%, the hydrolysis of gnemonoside A by glucosidase results in saturated condition of formed gnetin C which is slightly soluble in water, therefore the enzyme reaction stops. ***That is to say, these phenomena show that a difference in spot area between gnetin C (Rf 0.5) and gnemonoside A (Rf 0.15)*** on TLC ***represent the conversion of gnemonoside A into gnetin C.***

The results extracted and aged at room temperature (about 25°C) are shown in Table 2. Since the yields of the constituents of run 3 were the same as those of run 6 in Table 1, it is clear that low temperature elongates time of aging and the yield of gnetin C increases with prolonging time of the aging. This fact shows that aging proceeds during a long extraction by the sufficient action of glucosidase. No difference in the extraction and aging between ethanol and methanol can be detected. The extraction with only ethanol did not at all improve the yields of gnetin C by extending extraction time and heating to 80 °C.

Table 2. The influence of the concentration of ethanol and extraction temperature on aging at 25 °C

| run | concn. | time | yield | yield | | | | embodiment |
|---|---|---|---|---|---|---|---|---|
| | | d | extract | Gd. A[a] | Gd. D[b] | Gn. C[c] | sum[d] | |
| 1 | 16% | 16[e] | 10.1% | 0.28% | 0.14% | 0.11% | 0.52% | II-1 |
| 2 | 40% | 3 | 11.3% | 0.05% | 0.94% | 1.79% | 2.78% | II-9 |
| 3 | 50% | 2 | 9.3% | 0.47% | 0.70% | 1.48% | 2.65% | |
| 4 | 50%[f] | 5 | 11.3% | 0.03% | 0.46% | 1.67% | 2.16% | II-5 |
| 5 | 60% | 7 | 11.0% | 0.01% | 0.02% | 2.19% | 2.22% | II-3 |

a: gnemonoside A b: gnemonoside D c: gnetin C d: sumention G d: sum of Gd. A, Gd. D and Gn. C
e: hours f: methanol

When Indonesian Emping Belinjo brand snack food (like a cracker) is produced by heating endosperms, glucosidase is inactivated. The solution extracted by soaking the Emping with 50% ethanol at room temperature (25°C) for 2 days (Comparison I) showed the spot (Rf 0.15) on TLC and the peak on HPLC based on gnemonoside A, but gnemonoside D and gnetin C were not able to be detected. ***This result suggests that the endosperm contains only gnemonoside A which is converted into gnemonoside D and gnetin C by glucosidase hydrolysis.***

The above demonstrates clearly that the use of ***only polar-organic-solvent or water cannot get the extract*** contained gnetin C which shows ***both antimicrobial and antioxidative*** activities, but that of the mixture of both solvents and the control of temperature and time for the aging can improve solubility of the constituents and promote reactivity of glucosidase (namely, age) to obtain the extract containing rich gnetin C.

## Conclusion

The prior art does not disclose that the silbenoid constituent in the endosperm is gnemonoside A alone which is converted into gnetin C *via* gnemonoside D by the glucosidase in the endosperm. The claimed invention is the ***first time*** that the extraction of *Gnetum gnemon* seeds by soaking and aging in the ***mixture of polar organic solvent and water has*** resulted in the Gnetum extract contained rich gnetin C having ***both antimicrobial and antioxidative***

***actions.*** Therefore, the claimed invention is not *prima facie* obvious in light of the cited art which fails to disclose or even suggest the Gnetum extract now claimed.

In view of the above amendment, applicant believes the pending application is in condition for allowance.

The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 04-1105.

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Respectfully submitted,

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